Determination of Zearalenone in Corn: Collaborative Study

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Corn samples spiked at levels of 100, 300, 1000, and 2000 μg zearalenone/kg were sent to 22 collaborators for analysis by the Eppley method. All samples were yellow corn except one white corn sample spiked at 2000 μg/kg. Results from 16 collaborators were statistically analyzed. Only 4 of 16 collaborators detected zearalenone in the sample containing 100 μg/kg, but 11 detected the toxin in the sample containing 300 μg/kg. Average recoveries from all samples were 129% at 300 μg/kg, 101% at 1000 μg/kg, and 88% at 2000 μg/kg. The between-laboratory coefficients of variation were 53.0% at 300 μg/kg, 38.2% at 1000 μg/kg, and 27.0% at 2000 μg/kg. Five naturally contaminated corn samples, one in triplicate, were also provided. The mean level of zearalenone in the naturally contaminated samples ranged from 431 to 7622 μg/kg. The mean coefficient of variation for all samples was 40.5%. Two collaborators measured quantities of zearalenone on thin layer chromatographic plates densitometrically. Their results were not included in the statistical analysis, but the results indicated that densitometric measurement, given proper dilutions of solutions, could be used. The method has been adopted as official first action.

This study was conducted to determine whether a method developed by Eppley (1) for the screening of agricultural commodities for zearalenone, aflatoxin, and ochratoxin could be used to determine levels of zearalenone in white and yellow corn. The method, slightly modified, had been applied to the screening of 567 corn samples from commercial markets for the determination of zearalenone, aflatoxin, and ochratoxin (2, 3). The method had also been used to analyze 223 samples of the 1972 crop corn collected from terminal elevators or from stocks on hand at food processing establishments (4).

Collaborative Study

Description of Samples

Naturally contaminated lot samples of corn were ground to pass a U.S. standard No. 20 sieve, using a 6″ Raymond hammer mill equipped with a screen containing ⅛″ diameter round-hole perforations. Each ground sample (2–4 kg) was blended 15–30 min with a flat paddle at slow speed in a Hobart planetary mixer, Model A200, 12 qt capacity. All analytical samples (50 g) were preweighed into wide-mouth, 100 ml polyethylene bottles. Spiked samples were prepared by adding known amounts of zearalenone in benzene by syringe to preweighed portions of "clean" corn in individual bottles. The collaborators were instructed to use the entire contents of each bottle for analysis.

Description of Study

Twenty-two laboratories each received a practice sample with a noted level of zearalenone, 7 naturally contaminated samples, and 7 spiked samples. All but the practice sample were randomly coded. The samples of yellow corn were spiked to contain 100, 300, and 1000 μg/kg. The spiked white corn sample contained 2000 μg/kg. The laboratories were instructed to use all of the sample in a bottle for an analysis.

Zearalenone Reference Standard

Zearalenone dissolved in benzene was supplied in sealed ampoules; the concentration was 50 μg/ml. The ultraviolet absorption spectrum in methanol solution of the zearalenone used to prepare the standard solution was $\lambda_{max.}$ 314, 274, and 236 nm ($\varepsilon_{max.}$ 6240, 13,370, and 29,930). Reported values of ε at these wavelengths for crystalline zearalenone were 6000±5%, 13,900±5%, and 30,000±5% (5). The molecular absorptivity of zearalenone in benzene used to prepare the reference standard was found to be 6050 at 317 nm. Flame ionization gas chromatography of the trimethylsilyl derivative of the zearalenone used indicated a purity of >98%.

METHOD

ZEARALENONE

Corn—Official First Action

26.B01 ***Apparatus***

See **26.014**.

26.B02 ***Reagents***

See **26.002**, **26.015(a)** and **(b)**, and in addn:

(a) *Alcohol-chloroform mixt.*—5+95.

(b) *Aluminum chloride soln.*—Dissolve 20 g $AlCl_3 .6H_2O$ in 10 ml alcohol.

(c) *Zearalenone std soln.*—Det. chromatgc purity of cryst. zearalenone (available from Commercial Solvents Corp., Terre Haute, IN 47808) as in **26.011**. UV absorption in benzene: max. *A* 317 nm; ϵ 6060± 5%. UV absorption spectrum in MeOH: max. *A* 314, 274, and 236 nm; MW 318; ϵ 6000±5%, 13,900±5%, 30,000±5%, resp.; GLC purity of trimethylsilyl derivative >98%. Prep. soln contg 50 μg/ml benzene.

26.B03 ***Preparation of Sample***

Proceed as in **26.037**.

26.B04 ***Extraction***

Proceed as in **26.017(a)**.

26.B05 ***Column Chromatography***

(*Caution: See* **51.011**, **51.043**, **51.045**, **51.046**, and **51.061**.)

Prep. column, and add 50 ml $CHCl_3$ ext and 150 ml hexane wash as in **26.018(a)**. Wash column with 150 ml hexane and elute zearalenone with 250 ml acetone-benzene (5+95).

26.B06 ***Liquid-Liquid Partition***

Add few SiC chips to eluate contg zearalenone and evap. to near dryness on steam bath, preferably under gentle stream of N. Transfer residue to 60 ml separator with four 10 ml hexane washes. Finally, rinse with 10 ml CH_3CN and transfer to separator. Shake, and let phases sep. Sep. CH_3CN (lower) phase and ext hexane layer with 5 ml CH_3CN. Combine CH_3CN fractions and evap. to dryness in rotary vac. evaporator. Transfer to vial with $CHCl_3$. Evap., preferably under gentle stream of N. Seal with polyethylene stopper and cap. Save for TLC.

26.B07 ***Preparation of Plates for Thin Layer Chromatography***

Proceed as in **26.019(a)**, except that zearalenone replaces aflatoxin as test mycotoxin.

26.B08 ***Thin Layer Chromatography***

To residue, **26.B06**, add 500 μl benzene, seal with stopper, and shake vigorously on tube shaking machine to dissolve. For preliminary plate, apply 10 μl benzene soln to 2 spots. On one spot superimpose 5 μl zearalenone std soln, **26.B02(c)**, for internal std, and apply 5 μl zearalenone std soln to third spot.

Develop plate with alcohol-$CHCl_3$ (5+95), alcohol-$CHCl_3$ (3.5+96.5), HOAc-benzene (5+95), or HOAc-benzene (10+90), in lined, equilibrated tank ca 40 min. Compare spots presumed to be zearalenone with std. Zearalenone has greenish-blue fluorescence under shortwave UV (256 nm) at R_f ca 0.5 and is not visible under longwave UV light except at high concns. Examine sample spot contg internal std to verify identity of zearalenone. When presence of zearalenone is suspected, spray plates with $AlCl_3$ soln, heat 5 min at 130°, and examine under longwave UV light (365 nm). Zearalenone fluoresces blue under longwave UV light after spraying with $AlCl_3$ soln.

If zearalenone is detected in sample soln, perform quant. TLC. Spot 3, 5, and 7 μl zearalenone std soln and 4, 6, and 8 μl sample soln, and develop plate with alcohol-$CHCl_3$ (5+95) or other appropriate solvs as in par. 2. Compare fluorescent intensities of zearalenone spots of sample with those of std and det. which sample spot matches that of std. If spots of smallest portion of sample are too intense to match stds, dil. sample soln and rechromatograph.

26.B09 ***Calculations***

Calc. concn of zearalenone in μg/kg or ppb corn:

$$\mu g/kg = (S \times Y \times V)/(X \times W),$$

where S = μl zearalenone std soln equal to unknown; Y = concn of zearalenone std soln, μg/ml; V = μl of final diln of sample ext; X = μl sample ext spotted giving fluorescent intensity equal to S (zearalenone std soln); and W = g sample applied to column (10 g). If final ext diln does not represent 10 g, calc. correct sample wt and substitute.

Results and Discussion

The analytical results reported by 16 of the collaborators for the spiked corn samples are presented in Table 1; those for naturally contaminated corn are in Table 2. Two collaborators measured amounts of zearalenone on thin layer chromatographic (TLC) plates fluorodensitometrically, and their results are shown in Table 3.

Inspection of the study results (Tables 1 and 2) indicates that the Eppley method is suitable for determining zearalenone in corn. The limit of detection for the method is not much under 300 μg/kg, and one collaborator estimated that it was 250 μg/kg. The use of the aluminum chloride spray did not seem to increase the sensitivity. Only 4 of the 16 collaborators were able to detect zearalenone in the sample spiked at 100 μg/kg, and 5 did not detect the mycotoxin in the sample spiked at 300 μg/kg (Table 1). Recoveries were satisfactory at the levels at which zearalenone could be detected (300, 1000, and 2000 μg/kg).

Table 1. Collaborative results (μg zearalenone/kg sample) for visual analysis of zearalenone in spiked corn

Coll.	Sample 1[a] (0)	Sample 2 (100)	Sample 3 (300)	Sample 4 (1000)	Sample 5 (1000)	Sample 6 (1000)	Sample 7[b] (2000)
1	0	<125	(0)[c]	750	750	1709	1250
2	0	0	333	938	1250	938	2083
3	0	281	269	750	938	656	1880
4	1563	0	625	1333	833	625	833
5	0	0	375	938	938	1250	1250
6	0	0	100	800	625	625	1560
7	0	0	488	1300	1250	938	2500
8	0	trace	(0)	936	2000	900	1875
9	0	188	312	1000	875	875	1750
10	0	0	363	524	757	969	2200
11	0	0	(0)	624	417	625	1250
12	0	0	800	1500	1700	1700	2500
13	trace	trace	(4700)	1040	(0)	1560	1820
14	0	0	(0)		950	1250	1400
15	0	0	(0)	750	1000	750	2100
16	0	200	200	2000	800	1000	2000
Av.			386	1012	1005	1023	1766
Range: high			800	2000	2000	1709	2500
low			100	524	417	625	833
Std dev.			205	383	406	370	476
Coeff. of var., %			53.0	37.9	40.4	36.2	27.0
Av. rec., %			129	101	100	102	88.2
N[d]			10	15	15	16	16

[a] Values in parentheses under sample numbers are amounts (μg/kg) in sample.
[b] Spiked white corn sample.
[c] Values in parentheses were not included in calculations.
[d] N = number of values used to determine average.

In Tables 1 and 2, it is clear that the standard deviation increases as the mean level increases. When the same analyses were computed by using the logarithm of the zearalenone level, the standard deviation was more constant between samples. The logarithm transformation would be useful for a statistical analysis of zearalenone data with a wide range in values. A 2-way analysis of variance indicated that the variation between laboratories was significant at the 1% level. The overall precision estimates showed that the coefficient of variation on spiked samples was 31% within a laboratory and 44% between laboratories. The coefficient of variation for naturally contaminated samples within a laboratory was 35% and between laboratories, 52%. Differences in the means over all laboratories between the triplicate samples were not significant.

The least significant differences were calculated (6). The ratios of 2 values for spiked and naturally contaminated samples would have to be <2.16 and 2.31, respectively, within a laboratory to conclude that the results agreed. The respective ratios between laboratories would have to be 2.78 and 3.53. To attain a coefficient of variation or relative standard deviation of 20% based on the mean, at least 3 independent analyses of a given sample would have to be made. In the analysis of spiked corn samples, 64% of the variability is attributed to errors within the laboratory and 36% to between laboratories. For naturally contaminated samples, 51% of the variability of the analysis is caused by errors within the laboratory, and 49% by factors between laboratories.

Results obtained by the 2 investigators using fluorodensitometry to measure zearalenone on TLC plates are given in Table 3. Collaborator 18 listed his apparatus as a Zeiss; the excitation wavelength was 313 nm and fluorescence was measured at 443 nm. Collaborator 17 did not list his conditions. Also included in Table 3 are the results obtained at the Northern Regional Research Laboratory (NRRL) for the naturally contaminated corn samples, using the Eppley method (1) and measuring amounts of zearalenone on TLC plates densitometrically.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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Table 2. Collaborative results (μg zearalenone/kg sample) for visual analysis of zearalenone in naturally contaminated corn

Coll.	Sample 8	Sample 9	Sample 10[a]	Sample 11[a]	Sample 12[a]	Sample 13	Sample 14
1	375	375	938	886	917	2952	7500
2	333	1250	750	833	833	3125	7500
3	269	833	550	656	656	1690	7500
4	(0)[b]	1875	625	625	833	3125	10,000
5	500	938	938	625	938	3125	7813
6	200	312	470	468	312	1560	4050
7	488	938	938	1250	1250	3125	12,500
8	(0)	900	1550	(trace)	(trace)	2650	7300
9	625	750	875	750	750	2500	7500
10	398	732	534	570	535	1813	6583
11	125	312	312	417	625	937	4167
12	800	1100	1500	800	1000	3300	12,000
13	(0)	1000	1383	760	390	1380	2420
14	(0)	375	625	780	780	1875	7500
15	625	625	875	500	500	2500	10,000
16	250	800	400	400	400	800	6000
Av.	416	820	829	688	715	2278	7521
Range: high	800	1875	1550	1250	1250	3300	12,500
low	125	312	312	400	312	800	2420
Std dev.	199	400	379	219	260	842	2705
Coeff. of var., %	47.9	48.9	45.7	31.8	36.4	36.9	36.0
N[c]	12	16	16	15	15	16	16

[a] Triplicate series.
[b] Values in parentheses were not included in calculations.
[c] *N* = number of values used to determine average.

Table 3. Densitometric determination of zearalenone in corn

Spiked corn				Naturally contaminated corn				
	Added,	Found			Av.,[b]	Found		
Sample	μg/kg	Coll. 17	Coll. 18	Sample	μg/kg	Coll. 17	Coll. 18	NRRL[c]
1	0	0	0	8	431	275	500	
2	100	0	0	9	821	355	700	662
3	300	265	310	10[d]	858	435	900	815
4	1000	575	850	11[d]	708	685	900	
5	1000	750	1100	12[d]	737	435	900	
6	1000	850	1100	13	2377	1250	2400	1690
7	2000	1100	2200	14	7622	3000	5500	6850

[a] Excitation was measured at 313 nm, and fluorescence at 443 nm.
[b] As determined by other collaborators visually.
[c] After information was received from Collaborator 18, samples were assayed at NRRL densitometrically.
[d] Triplicate series.

To determine the effect of substances in corn extracts on visual and densitometric measurements of zearalenone zones, partially purified extracts of zearalenone-free corn were prepared for TLC by the Eppley method. Residues of the extracts were spiked before TLC with quantities of crystalline zearalenone in benzene to represent different levels of contamination. After development, the TLC plates were read both visually and densitometrically; *see* Table 4. Results obtained densitometrically were more consistent than those obtained visually.

Collaborator 9 suggested that the liquid-liquid partition step with acetonitrile-hexane be omitted initially to save time. The partition would be performed to obtain cleaner extracts only if preliminary TLC indicated that zearalenone was present. Collaborator 12 did not think the liquid-liquid partition was effective in removing impurities from the extracts.

Most of the comments concerned the TLC step. Four collaborators suggested either applying less zearalenone standard solution to the plates and in smaller increments or diluting the standard. Concentrations mentioned for the standard were 10 or 25–30 μg zearalenone/ml. The collaborators reported that acetic acid-benzene (10+90), ethanol-chloroform (3.5+

Table 4. Effect of substances in corn extracts on determination of zearalenone levels (μg/kg)[a]

Level in original corn represented by spiked ext	Levels determined on TLC plates	
	Visually	Densitometrically
300	625	327
	625	298
500	703	424
	625	504
1000	1094	730
	1250	850
3000	4375	2690
	2500	2615
5000	5410	5445
	5000	4850
8000	10,000	9545
		9155

[a] Excitation was measured at 313 nm, and fluorescence at 443 nm.

96.5), or acetic acid-benzene (5+95) were also effective solvent systems. Other than the Adsorbosil-1 plates described, the following TLC plates were used with success by one or more collaborators: precoated kieselgel G (0.25 mm), precoated Brinkmann Silplate-22 (0.25 mm), precoated Brinkmann G-25-HR (No. 6614600-6), Mallinckrodt-7G, and precoated Merck silica gel 60 plates.

Recommendation

Results of the collaborative study indicate that the Eppley method, modified as described in this report, is applicable to the determination of zearalenone in corn. It is recommended that the modified method (1; R. M. Eppley, 1968, Food and Drug Administration, Washington, DC) be adopted as official first action for the determination of zearalenone in corn.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C and was adopted by the Association. *See* (1976) *JAOAC* **59**, 386–387.

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